Fucoidan interferes with Porphyromonas gingivalis-induced aneurysm enlargement by decreasing neutrophil activation

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Purpose: **Neutrophils have been shown to be involved in all stages of human and experimental abdominal aortic aneurysm (AAA) development. The initial processes of neutrophil rolling and trapping in the intraluminal thrombus (ILT) are mediated mainly by P-selectin expressed by activated platelets. In the present study, we propose to evaluate the beneficial effect of fucoidan, a competitive binding agent of P-selectin, on aneurysmal growth in a rat model of aortic aneurysm with neutrophil enrichment of the ILT induced by repeated episodes of weak bacteremia.**

Methods: **Sixty Lewis rats with experimental AAAs, developed from decellularized aortic xenografts, were divided into four groups. Two groups were used as controls: group fucoidan control (FC) was treated with 200 mg of fucoidan (F) delivered by 2 mL, 4-week osmotic pumps placed intraperitoneally before closing the abdomen, and group C received saline instead of fucoidan. Two more groups were injected weekly with Porphyromonas gingivalis (*P. gingivalis* [Pg]): group F+Pg received 200 mg of intraperitoneal fucoidan and group Pg received saline. AAAs were harvested after 4 weeks and peripheral blood was sampled at that time. Cell-free DNA (cf-DNA) and myeloperoxydase (MPO) antigen concentrations were determined in plasma and in AAA-conditioned media. Histology and P-selectin immunostaining were performed on AAA tissue samples.**

Results: **Comparing rats injected with Pg, those receiving fucoidan presented reduced aneurysmal diameter. Histologic analysis of AAAs showed that fucoidan reduced the ILT thickness in Pg-injected rats, with fewer trapped neutrophils, and with signs of a healing process, as observed in control group C. Immunohistological analysis revealed a substantial decrease in P-selectin immunostaining at the luminal surface of aneurysms in fucoidan-treated rats compared to the other groups, suggesting an interaction between fucoidan and P-selectin. A significant decrease in MPO concentrations in both plasma and conditioned medium was induced by fucoidan treatment in Pg-injected rats, reflecting a pacification of the ILT biological activity. This effect was associated with a reduction in neutrophil activation and apoptosis, reflected by a significant decrease in cf-DNA concentration in both plasma and conditioned medium of fucoidan-treated rats.**

Conclusions: **Our results suggest that fucoidan has a beneficial effect on experimental aneurysmal degeneration by decreasing neutrophil activation in the ILT enhanced by weak pathogen contamination. This effect seems to be related to its interaction with P-selectin, which may decrease the trapping of neutrophils into the ILT. Fucoidan could represent a therapeutic option in AAAs to decrease the neutrophil activation involved in the degenerative process of aneurysmal expansion and rupture. (J Vasc Surg 2013;57:796-805.)**

Clinical Relevance: **In the present study, we propose to evaluate the beneficial effect of fucoidan, a competitive binding agent of P-selectin, on aneurysmal growth in a rat model of aortic aneurysm with neutrophil enrichment of the intraluminal thrombus induced by repeated episodes of weak bacteremia. Our results suggest that fucoidan has a beneficial effect on experimental aneurysmal degeneration enhanced by weak pathogen contamination by decreasing the trapping of neutrophils and the release of proteolytic enzymes into the intraluminal thrombus. Fucoidan could, therefore, represent a therapeutic option in abdominal aortic aneurysm to decrease the degenerative process leading to aneurysmal expansion and rupture.**

Neutrophils have been shown to be involved in the early and late stages of human and experimental abdominal aortic aneurysm (AAA) development.[1,2] Their adhesion to intraluminal thrombus (ILT) prevents ILT colonization by adherent mesenchymal cells and subsequent healing,[3] and neutrophil extravasation is an important component of aneurysmal dilation.[4,5] Biological interactions between bacteria and thrombus formation have recently been emphasized.[6]

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Moreover, we demonstrated in a recent study that a periodontal pathogen (Porphyromonas gingivalis [*P. gingivalis*]) enhances the development of AAAs in a rat model by promoting the presence of a neutrophil-rich ILT, leading to a pathophysiological pattern similar to that observed in humans.[7]

Neutrophil recruitment in the ILT is a multistep process that typically consists of initial neutrophil rolling on activated platelets, leading to their trapping in the platelet-rich fibrin network,[8] and their subsequent death, causing release of the proteolytic content of their granules.[9,10] The initial processes of neutrophil rolling and trapping in ILT are mediated mainly by P-selectin expressed by activated platelets[11,12] and its counter receptor the P-selectin glycoprotein ligand-1 on the neutrophil surface.[13,14]

Early studies,[15-18] and a recent study by our group,[19] have demonstrated the high affinity of fucoidan for P-selectin, as well as its ability to bind to the platelet-rich ILT in vivo.[20] Fucoidan has been reported to prevent selectin-dependent recruitment of leukocytes by the activated endothelium in experimental ischemia-reperfusion (Morya et al[21] for review). Nevertheless, the in vivo potential beneficial effect of fucoidan on leukocyte-thrombus interactions has never been tested. In the present study, we propose to evaluate the potential benefit of fucoidan as a competitive binding agent of P-selectin, and to assess its effect on aneurysmal growth in a recently described rat model of aortic aneurysm characterized by a neutrophil-rich ILT induced by repeated episodes of weak bacteremia.[7]

METHODS

Experimental model of AAA. Experimental AAAs were induced by implanting a segment of decellularized guinea pig aorta in the rat as previously described.[22] Briefly, under intraperitoneal pentobarbital anesthesia, guinea pig infrarenal aortas (1.5 cm) were sampled and decellularized by sodium dodecyl sulfate treatment (0.1%; overnight 4°C). After washing in saline, aortas were orthotopically transplanted into the abdominal aorta of the Lewis rat. Postoperatively, animals were fed a standard diet and their care complied with the principles of Laboratory Animal Care formulated by the European Union. This experimentation was carried out under the authority of the French Ministry of Agriculture (authorization n° 75-214, delivered March 25, 2003). This model is characterized by an initial immune injury of the xenogenic extracellular matrix,[22] and a secondary spontaneous healing process, which can be accelerated by smooth muscle cells seeding.[23]

***P. gingivalis* culture.** *P. gingivalis* strain was purchased from the Collection de l'Institut Pasteur (Paris, France) and was grown on M20 medium, consisting of 3% (w/v) tryptone, 1.5% (w/v) agar, 2% (w/v) yeast extract, 0.05% (w/v) cysteine hydrochloride, 05% (w/v) glucose, 2.5% (v/v) hemin solution (0.1% (w/v) hemin chloride, 4% (v/v) triethanolamine), in an anaerobic chamber at 37°C (Bio-Merieux, Lyon, France). Bacteria were subcultured once a week. A total of 1 mL of the cell suspension was centrifuged (5000 *g*, 5 minutes) and resuspended in 12 mL of M20 medium. Weekly injections of *P. gingivalis* in the above-described model prevent the spontaneous healing process.[7]

Experimental study design. Sixty male Lewis rats and 60 guinea pigs were used for this study. Simultaneously with transplantation (D0), the rats were randomly allocated to:

- **Group C** (control) who received 2 mL of saline via a 2 mL, 4-week osmotic pump (Model 2ML4, Alzet, Cupertino, Calif) placed intraperitoneally, immediately after xenografting, before closing the abdomen.
- **Group Pg** (*P. gingivalis*) who received 2 mL of saline in a 2 mL, 4-week osmotic pump as in group C and *P. gingivalis* (10^7 colony forming units [CFUs]/500 μL/rat), injected once a week via the jugular vein for 4 weeks.
- **Group FC** (fucoidan control) who received 200 mg of fucoidan in a 2 mL, 4-week osmotic pump placed intraperitoneally.
- **Group F+Pg** (fucoidan + *P. gingivalis*) who received 200 mg of fucoidan in a 2 mL, 4-week osmotic pump as for group FC and *P. gingivalis* (10^7 CFU/500 μL/rat) injected once a week for 4 weeks as for group Pg.

After 4 weeks of treatment, rats were anesthetized by intraperitoneal pentobarbital injection. Blood was collected in citrated tubes; the maximal diameter of the AAA was measured in vivo using a grid in the microscope eyepiece before its removal for further study. For each animal, the harvested aneurysmal tissue was divided in two parts: one for histologic analysis and one for biochemical analysis on conditioned media. The aneurysmal wall was fixed in paraformaldehyde (3.7%) for immunohistochemical analysis or incubated for 24 hours in Roswell Park Memorial Institute medium−1640 at 37°C (Invitrogen, Cergy-Pontoise, France) (6 mL/g of wet tissue), in order to obtain conditioned medium. The conditioned medium was centrifuged at 3000 *g* for 10 minutes at 20°C and the supernatant was then aliquoted and stored at −80°C until use.

Plasma neutrophil counts. Neutrophils were quantified in peripheral blood using a Scil Vet ABC animal blood counter (SCIL Animal Care Company GmbH, Viernheim, Germany). Counts were expressed as $\times 10^3/\mu L$ of blood.

Determination of cell-free DNA concentrations. Cell-free DNA (cf-DNA) concentration was determined in the conditioned medium of experimental AAA samples and in rat plasma, using Quant-it Picogreen dsDNA Reagent (Invitrogen). Briefly, 10 μL of samples and Lambda DNA standard (1 ng/mL −1 μg/mL) were diluted in TE buffer (200 mM Tris-HCl, 20 m*M* EDTA, pH7.5, 100-μL final) before addition of 100 μL Picogreen dsDNA reagent. After mixing and incubation for 5 minutes at room temperature in the dark, the fluorescence was measured using a microplate reader (excitation 480 nm, emission 520 nm).

Determination of myeloperoxidase antigen concentrations. The concentration of myeloperoxidase (MPO) antigen, a marker of neutrophil activation,[10] in conditioned medium and in plasma of rats was determined using the rat

MPO enzyme linked immunosorbent assay kit from Hycult Biotechnology (Uden, The Netherlands).

Histology and immunostaining. After euthanization (28 days), relevant tissue of each samples were fixed in paraformaldehyde for 24 hours, embedded in paraffin, and cut into 5-μm sections for morphologic analysis. Serial sections were stained with Masson trichrome and hematoxylin & eosin to visualize cells, nuclei and fibrin, and with orcein to show elastin fibers and internal elastic lamina. Immunostaining was performed on deparaffinized sections incubated with a primary goat antimouse P-selectin antibody (SC 6943; Santa Cruz Biotechnology, Santa Cruz, Calif) (1/50) revealed by a secondary rabbit antigoat immunoglobulin G (Dako P0160; Dako North America, Carpinteria, Calif) (1/200), followed by the 3,3-diaminobenzidine reaction, and counterstaining with Mayer hematoxylin.

Immunofluorescence. The 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (100 ng/mL) was added for 15 minutes on deparaffinized sections and slides were mounted using Fluoprep mounting medium (Dako) to visualize nuclear, and extranuclear DNA. Primary monoclonal antibody anti-α-smooth muscle actin (1/60; α-SMA; Dako) was used to visualize smooth muscle cells of mesenchymal cells in the aortic wall.

Statistical analysis. The study was designed with 80% power to detect a relative 50% difference in aneurysmal size between *P. gingivalis* and fucoidan + *P. gingivalis* groups. Statistical testing was performed at the two-tailed (alpha) level of 0.05 using a nonparametric Mann-Whitney U test. Based on preliminary data indicating that the average aneurysmal size of Pg-injected rats at 5 weeks after xenograft surgery was 7.62 mm, SD: 1.4, we used 15 rats for each group (Saline; Pg; fucoidan + saline; fucoidan + Pg). A computer-based randomization was used to allocate fucoidan or saline pump and *P. gingivalis* or saline injection to each rat.

Results are expressed as box plots in which the boxes represent the 25th and 75th percentiles, the line within the box represents the median value, and the lines outside the boxes represent the 5th and the 95th percentiles. Comparisons between groups were performed by two-way analysis of variance (ANOVA) as appropriate, followed by a post hoc Bonferroni test (Prism 5; GraphPad Software Inc, San Diego, Calif) for intergroup pairwise comparisons. Statistical significance was accepted when $P < .05$.

RESULTS

Repeated episodes of *P. gingivalis* bacteremia induce neutrophil activation and aneurysmal growth. In order to confirm that *P. gingivalis* bacteremia impacts aneurysmal progression, we used an experimental xenograft-induced model of aneurysm in the rat. To mimic the transitory repeated bacteremia associated with periodontal disease, *P. gingivalis* was injected intravenously once a week for 4 weeks (10^7 CFU/rat). *P. gingivalis* injections induced a significant increase in aneurysm maximal diameter as compared to saline-injected control rats (Fig 1; group Pg: 6.459 ± 1.789 mm vs group C: 4.027 ± 1.485 mm; $P < .001$).

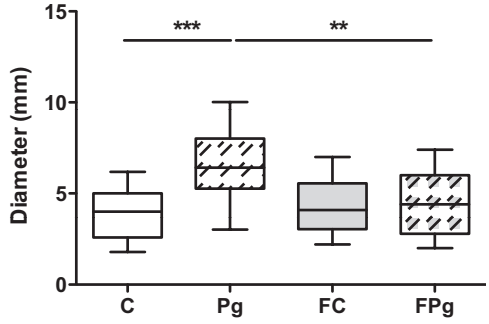

Fig 1. Intraperitoneal fucoidan release inhibits the aneurysmal growth induced by Porphyromonas gingivalis (*P. gingivalis* [*Pg*]) injections. Maximal abdominal aortic aneurysm (AAA) diameters in each group are presented as box plots in which the median is shown. $^{**}P < .01$ and $^{***}P < .001$ (one-way analysis of variance [ANOVA], Bonferroni test). *C,* Control; *FC,* fucoidan control.

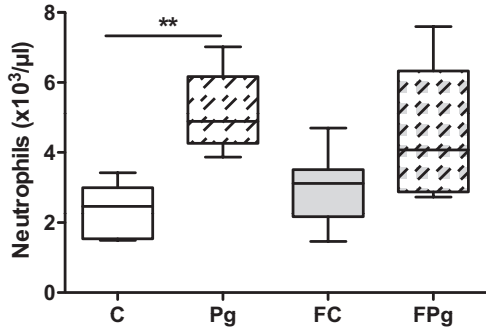

Fig 2. Circulating neutrophils were significantly increased in rats injected weekly with Porphyromonas gingivalis (*P. gingivalis* [*Pg*]). $^{**}P < .01$ (one-way analysis of variance [ANOVA], Bonferroni test). *C,* Control; *FC,* fucoidan control.

Circulating neutrophils were significantly increased in blood samples of rats injected weekly with *P. gingivalis* compared to group C (Fig 2; group Pg: 5.881 ± 1.593 vs group C: 2.123 ± 1.123; $P < .05$). This effect of *P. gingivalis* bacteremia on circulating neutrophils was independent of fucoidan treatment, as shown by the lack of interaction in two-way ANOVA analysis (Table).

To further demonstrate neutrophil activation in the aneurysmal wall of *P. gingivalis* injected rats, MPO concentrations were measured in plasma and in the conditioned medium. The MPO concentration was strongly increased in plasma and in AAA-conditioned medium of *P. gingivalis*-injected rats compared to those of noninjected rats (Fig 3, *A*; in plasma; group Pg: 1764 ± 57.76 vs group C: 1282 ± 150.3; $P < .001$; Fig 3, *B*; in conditioned medium; group Pg: 1456 ± 150.6 vs group C: 892.2 ± 77.56; $P < .001$).

Moreover, cf-DNA concentration was increased in both plasma and conditioned medium of rats having re-

Table. Statistical values: the dependence of the effect of fucoidan on Porphyromonas gingivalis bacteremia was evaluated by two-way ANOVA analysis

Two-way ANOVA	*Fucoidan*	*Pg*	*Interaction*
AAA diameter	f = 5.01 $P = .029$	f = 11.87 $P = .0011$	f = 9.43 $P < .01$
Circulating neutrophils	f = 0.02 $P = .87$	f = 19.95 $P < .001$	f = 1.45 NS
MPO Ag in plasma	f = 28.39 $P < .0001$	f = 38.10 $P < .0001$	f = 18.20 $P < .001$
MPO Ag in conditioned media	f = 12.79 $P < .001$	f = 103.75 $P < .0001$	f = 24.75 $P < .0001$
Cf-DNA in plasma	f = 8.39 $P < .01$	f = 86.87 $P < .0001$	f = 3.62 NS
Cf-DNA in conditioned media	f = 1.68 $P < .05$	f = 7.17 $P < .01$	f = 7.51 $P < .01$

AAA, Aortic abdominal aneurysm; *ANOVA,* analysis of variance; *cf-DNA,* cell-free DNA concentration; *MPO Ag,* myeloperoxydase antigen concentration; *Pg,* Porphyromonas gingivalis.

ceived *P. gingivalis* (Fig 4, *A*; in plasma; group Pg: 1080 ± 190 vs group C: 492.3 ± 190.3; $P < .001$; Fig 4, *B*; in conditioned medium; group Pg: 5853 ± 500 vs group C: 4481 ± 1101; $P < .01$).

Histologic analysis of Pg-injected AAAs confirmed the thrombus formation on Masson Trichrome staining (Fig 5, *A*), inside a degraded internal elastic lamina on orcein staining (Fig 6, *A*), with an enrichment of neutrophil polynuclear cells at the luminal layer of the ILT on hematoxylin & eosin staining (Fig 6, *C*) and on DAPI immunofluorescence (Fig 7, *A*). Cf-DNA probably corresponding to neutrophil extracellular traps (NETs) could also be visualized in deeper layers of the ILT (Fig 7, *E*), signing a neutrophil activation in the ILT of Pg injected experimental AAA.

Intraperitoneal fucoidan treatment inhibits neutrophil activation and aneurysmal growth induced by *P. gingivalis* bacteremia. In order to provide a proof of concept that fucoidan may inhibit thrombus/neutrophil/bacteria interactions, we inserted an intraperitoneal osmotic pump that continuously released 200 mg of fucoidan throughout the 4 weeks of the experiment. This treatment by fucoidan had no significant impact on noninjected AAA diameter compared to controls (Fig 1; group F: 4.343 ± 1.645 mm vs group C: 4.027 ± 1.485 mm; $P > .5$), circulating neutrophil level (Fig 2; group F: 2067 ± 0.5437 vs group C: 2123 ± 1123; $P > .5$), MPO concentrations (Fig 3, *A*; in plasma; group F: 1233 ± 99.30 vs group C: 1282 ± 150.3; $P > .5$; Fig 3, *B*; in conditioned medium; group F: 944.2 ± 75.15 vs group C: 892.2 ± 77.56; $P > .5$), or cf-DNA (Fig 4, *A*; in plasma; group F: 440.3 ± 103.8 vs group C: 492.3 ± 190.3; $P > .5$; Fig 4, *B*; in conditioned medium; group F: 4847 ± 1386 vs group C: 4481 ± 1101; $P > .5$).

In the two groups of *P. gingivalis*-injected rats, those receiving 200 mg of fucoidan over the experimental period of 4 weeks showed smaller aneurysmal diameters, reflecting inhibition of aneurysmal growth (Fig 1; group F+Pg: 4480 ± 1645 mm vs group Pg: 6.459 ± 1.789 mm; $P < .01$). This effect of fucoidan on AAA diameter was strongly dependent on *P. gingivalis* bacteremia, as shown by a significant interaction in the two-way ANOVA analysis (Table).

Histologic analysis showed that fucoidan reduced the ILT thickness in AAAs of Pg-injected rats (Fig 6, *A* and *B*), with fewer trapped neutrophils (Fig 6, *D*), preservation of the elastic fiber network (Fig 6, *A* and *B*), and signs of a healing process (ie, colonization by α-actin positive mesenchymal cells as observed in immunofluorescence) (Fig 7, *D*). Fucoidan also seemed to induce a substantial decrease in P-selectin immunostaining at the luminal surface of ILT compared to the Pg alone (Fig 6, *E* and *F*).

A strong significant decrease in MPO concentrations in both plasma and conditioned medium was induced by fucoidan treatment in Pg-injected rats, reflecting a pacification of biological activity in the ILT (Fig 3, *A*; in plasma; group Pg: 1764 ± 57.76 vs group F+Pg: 1321 ± 180.1; $P < .001$; Fig 3, *B*; in conditioned medium; group Pg: 1456 ± 150.6 vs group F+Pg: 1138 ± 97.97; $P < .001$). This effect of fucoidan on MPO concentrations, reflecting a decrease in neutrophil activation in the conditioned medium and in plasma, was strongly dependent on *P. gingivalis* bacteremia, as shown by a significant interaction in the two-way ANOVA analysis (Table).

This effect was associated with a reduction in neutrophil recruitment and apoptosis, reflected by a significant decrease in cf-DNA concentrations in both plasma and conditioned medium of fucoidan-treated rats (Fig 4, *A*; in plasma; group Pg: 1080 ± 189.7 vs group F+Pg: 828.7 ± 231.7; $P < .05$; Fig 4, *B*; in conditioned medium; group Pg: 5853 ± 499.7 vs group F+Pg: 4838 ± 824.1; $P < .05$). An interaction between fucoidan treatment and *P. gingivalis* bacteremia was found to be significant by two-way ANOVA analysis of cf-DNA concentrations in conditioned media, but did not reach significance in plasma (Table). Extra nuclear DNA could also be visualized into the ILT of Pg injected AAA on DAPI staining, whereas only nuclear DNA was detected after fucoidan treatment (Fig 7, *E* and *F*).

DISCUSSION

This study shows that the intraperitoneal perfusion of 200 mg of fucoidan over a period of 4 weeks prevented the ILT-dependent enhancement of AAA dilatation induced by weekly repeated episodes of *P. gingivalis* bacteremia in a rat experimental model. This impact of fucoidan seems to be related to an interaction with P-selectin, which decreases neutrophils activation, and the release of their proteolytic enzymes and cf-DNA into the ILT. This could represent a therapeutic option in order to decrease the degenerative process of aneurysmal expansion, which inevitably leads to rupture in patients with an atherothrombotic AAA involving neutrophil recruitment.[23]

We developed a model of arterial aneurysm formation by taking advantage of the immunogenicity of guinea pig aortic extracellular matrix grafted into

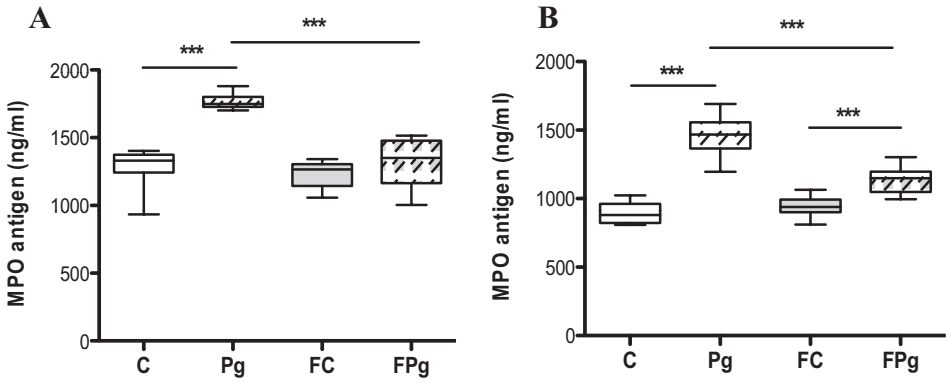

Fig 3. The increased myeloperoxydase (*MPO*) concentrations in plasma **(A)** and in abdominal aortic aneurysm (AAA)-conditioned media **(B)** induced by Porphyromonas gingivalis (*P. gingivalis* [*Pg*]) injections were significantly inhibited by intraperitoneal administration of fucoidan. Results are presented as box plots with median. ***$P < .001$ (one-way analysis of variance [ANOVA], Bonferroni test). *C,* Control; *FC,* fucoidan control.

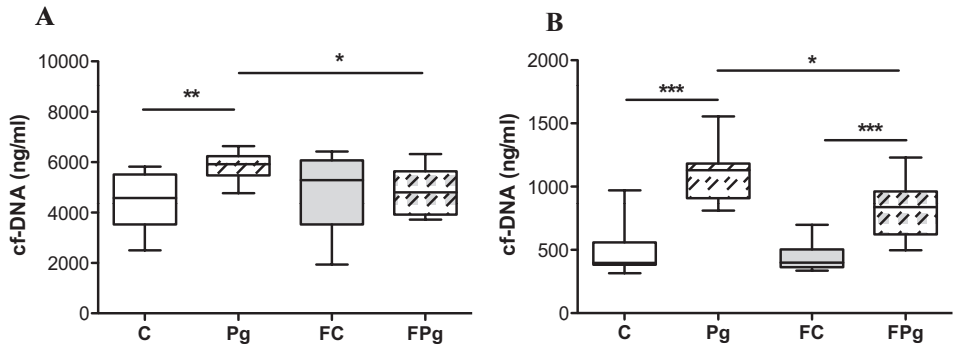

Fig 4. Increased cell-free DNA (*cf-DNA*) in plasma **(A)** and abdominal aortic aneurysm (AAA)-conditioned media **(B)** of Porphyromonas gingivalis (*P. gingivalis* [*Pg*])-injected rats was significantly inhibited by intraperitoneal fucoidan release. Results are presented as box plots with median. *$P < .05$, **$P < .01$, and ***$P < .001$ (one-way analysis of variance [ANOVA], Bonferroni test). *C,* Control; *FC,* fucoidan control.

rats.[22,24] During the rejection of the xenografted extracellular matrix, monocyte/macrophages and T lymphocytes penetrate into the graft media where immunoglobulins are deposited. Elastin in the media is degraded and the graft dilates and becomes aneurysmal by 1 month after engraftment.[22] During aneurysm formation in this model, the gelatinolytic and elastinolytic matrix metalloproteinase (MMP)-9 is upregulated.[25] In this respect, this xenograft model reproduces the main features of the human disease (arterial dilation and rupture, elastin degradation, inflammatory cell infiltration in the media, and upregulation of MMPs). This animal model is characterized by the formation of a thrombus, about 1 week after grafting the decellularized guinea pig aorta in the abdominal position in rats and associated with aortic dilation. In this model, like in all currently used AAA models, in the absence of additional aggression the mural thrombus is colonized by mesenchymal cells, initiating a healing process. In contrast, after 4 weekly intravenous injections of *P. gingivalis,* the aortic diameter was not only increased relative to saline-injected rats, but the composition of the AAA wall was also strikingly different. In *P. gingivalis*-injected rats, the mural ILT was persistent and exhibited a multilayered aspect, similar to that observed in human AAA. The ILT was considerably enriched in neutrophils and all markers of their activation were increased in conditioned medium and in plasma of *P. gingivalis*-injected vs saline-injected rats.

In a previous study, we have demonstrated that stimulation of neutrophils by *P. gingivalis* led to NETs production, reflected by an increased cf-DNA concentration in the supernatant.[7] The presence of NETs in AAA samples was also associated with a release of cf-DNA measurable in conditioned medium (thrombus and adventitia) and increased in plasma of patients with AAAs relative to control subjects. In addition, concentration of cf-DNA was positively correlated with the abdominal aortic diameter, suggesting that this marker was a good reflection of the neutrophil activity in the thrombus. Neutrophils entrapped in the luminal pole of the ILT of AAAs release proteinases such as MMP-9 and MPO and elastase. Fontaine et al[3]

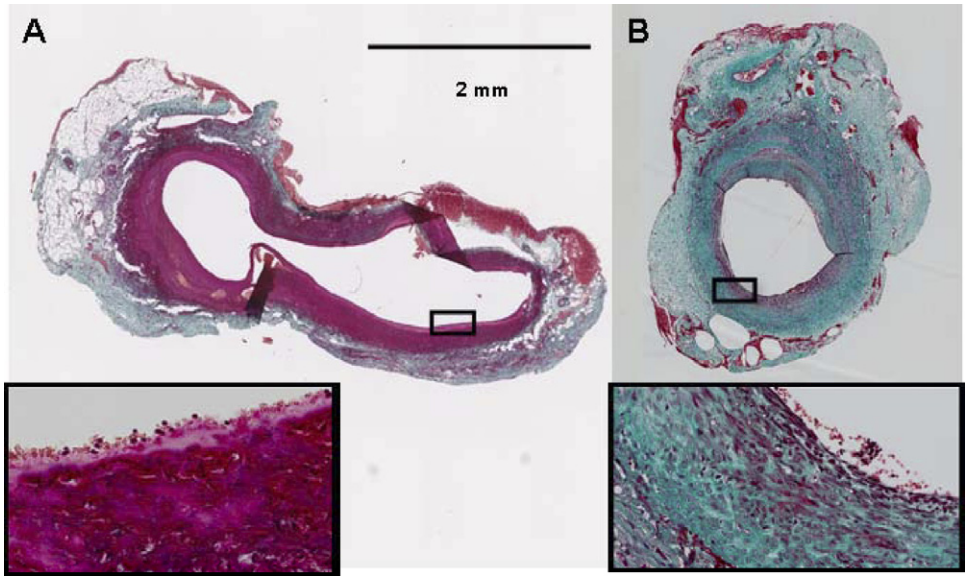

Fig 5. After fixing in paraformaldehyde, abdominal aortic aneurysm (AAA) samples of group Porphyromonas gingivalis (*P. gingivalis*; Pg) **(A)** and group fucoidan (F)+Pg **(B)** were embedded in paraffin and sectioned at 5 μm for histology. Slides were stained with Masson Trichrome and analyzed at ×1.25 and ×40, as presented. **A,** Pg injections induced neutrophil recruitment, thrombus enlargement, and inhibited healing. **B,** Fucoidan reduced the intraluminal thrombus (ILT) thickness in Pg-injected rats, with less neutrophil trapping, and organizing thrombus.

provided the evidence that neutrophil elastase adsorbed in the fibrin matrix can prevent recolonization of the thrombus by both smooth muscle cells and mesenchymal cells. These data pointed to new therapeutic strategies involving the possible use of neutrophil activation inhibitors allowing cellular healing of ILT, therefore, preventing aneurysm evolution.

Several lines of evidence led us to hypothesize that bacteria and particular periodontal pathogens may participate in the development of AAAs. Epidemiologic data suggest a potential association between periodontal and cardiovascular diseases.[26] The nature of this association is, however, still debated; in particular, whether periodontal disease impacts directly on the pathogenesis of cardiovascular disease or indirectly by increasing inflammation background is not settled. Interestingly, atherothrombosis and periodontal diseases share risk factors such as age, gender, hypertension, and smoking. Gender, age, and smoking are the most important risk factors for AAAs.[27,28] Heretofore, no epidemiologic study has been performed potentially linking AAA development and periodontal disease.

As previously described, this *P. gingivalis* periodontal pathogen enhances the development of AAAs by maintaining the presence of a neutrophil-rich ILT, leading to a pathophysiological pattern similar to that observed in humans.[7] We believe that this experimental model provides the opportunity to test new therapeutic agents specifically directed against neutrophil binding and activation within the ILT. Antibiotics such as doxycycline have already shown a potential impact on aneurysmal stabilization, due, at least in part, to neutrophil rarefaction.[29,30] In one of our preceding studies,[3] we demonstrated that neutrophils represent the major impediment to ILT recolonization by mesenchymal cells in human AAA. Therefore, characterizing agents that inhibit neutrophil interactions with platelet-rich fibrin and their subsequent trapping in the ILT could be of great interest to promote the healing process in the aneurysmal vascular wall. We and others have pointed out the potential mediators of neutrophil recruitment, such as L-selectin and P-selectin,[31,32] and chemoattractants such as regulated on activation, normal T expressed and secreted cytokine (RANTES), interleukin-8, and leukotriene B4.[33] In the cardiovascular context, P-selectin expression is involved in the pathophysiology of the renewal and growth of biologically active thrombi in arterial atherothrombotic plaques.[8] P-selectin promotes interactions between platelets, bacteria, and leukocytes,[6] representing an important molecular target in the regulation of thrombus-dependent aortic wall degenerative processes.

Many studies have shown that mimics of sialyl Lewis X (SLeX), oligosaccharides, and sulfated polysaccharides such as heparan and fucoidan, or some of their derivatives, are able to interact with P-selectin.[13,29,34] Fucoidan, a type of sulfated polysaccharide derived from brown seaweed, is a naturally occurring mimic of SLeX,[35] which is the natural ligand of P-selectin.[36] Our group recently demonstrated the high affinity of fucoidan for P-selectin,[19] as well as its low nonspecific binding.[20] We showed that low molecular weight fucoidan prevented P-selectin binding to SLeX with nanomolar concentration range. Moreover, the binding of fucoidan to human platelets increased with the level of

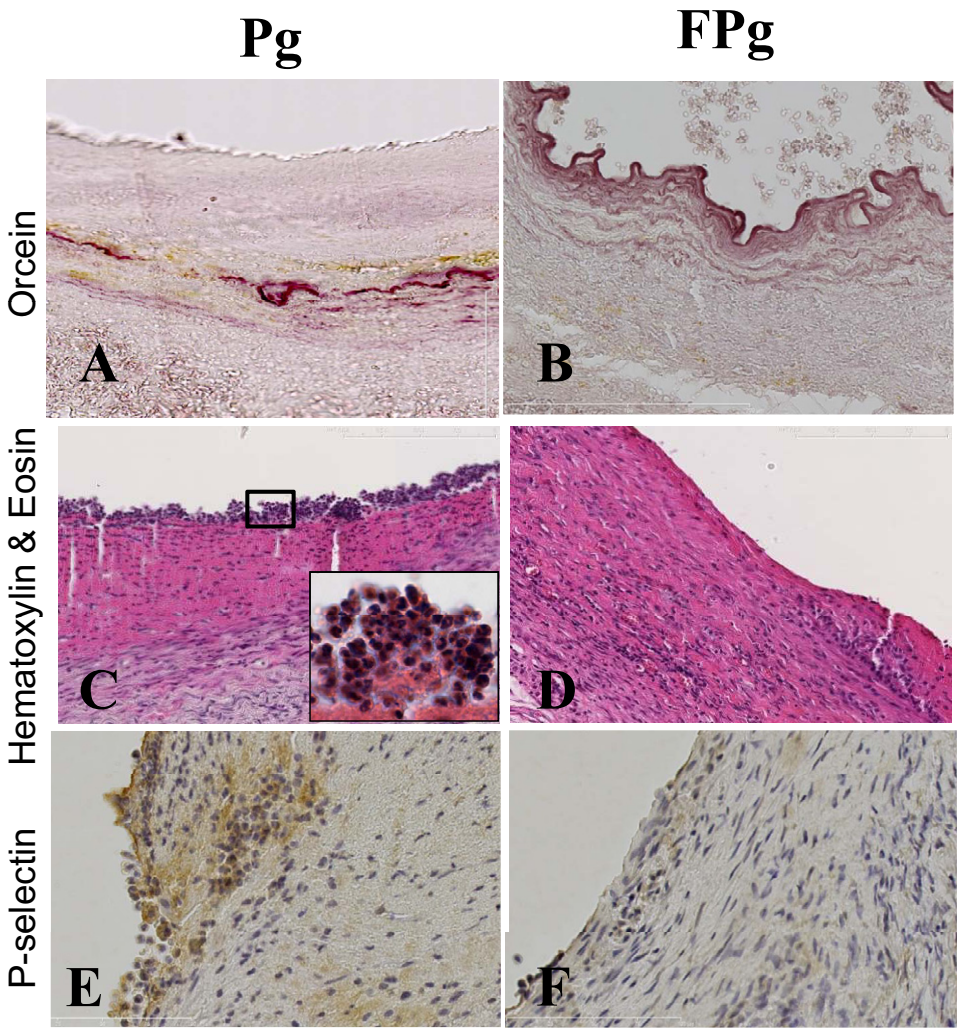

Fig 6. A preservation of the elastic fiber network is induced by fucoidan on orcein-stained slides at ×40 **(B)**, and thrombus can be seen inside the degraded internal elastic lamina in the Porphyromonas gingivalis (*P. gingivalis*; *Pg*) group **(A)**. A qualitative indication of neutrophil increased number in abdominal aortic aneurysm (AAA) sections of the Pg group compared to the fucoidan (F)+Pg group is given by hematoxylin & eosin staining at ×20 and ×100 **(C and D)**. P-selectin immunostaining was decreased at the luminal surface of aneurysmal aortic walls in fucoidan-treated rats, suggesting an interaction between fucoidan and P-selectin. P-selectin immunostaining of the aortic wall of AAAs of group Pg **(E)**, and group F+Pg **(F)** are presented with hematoxylin nuclear counterstaining ×63.

platelet activation, and the binding of anti–P-selectin antibody to activated platelets was inhibited by fucoidan.[19] Therefore, we used fucoidan-containing compound as a contrast agent for imaging ILT in experimental models of AAA in rats.[20] These data suggested the ability of fucoidan to target P-selectin in ILT.

Fucoidan seems to have a localized action on neutrophil activation in the ILT, at the level of the AAA, as a strong interaction between fucoidan treatment and *P. gingivalis*-induced neutrophil activation could be demonstrated on AAA diameter, MPO concentrations in both conditioned media and plasma, as on cf-DNA in conditioned media, but not on circulating neutrophil counts or on cf-DNA concentrations in plasma, suggesting a more specific action of fucoidan at the level of the ILT rather than a systemic effect on neutrophils.

Fucoidan is also able to inhibit adhesion of some bacterial species to endogenous glycoproteins, such as *Escherichia coli*,[37] *Streptococcus*,[38] *Helicobacter pylori*,[39] and *Staphylococcus*,[40] and therefore to limit their infectious invasiveness. An effect of fucoidan on *P. gingivalis* has never been reported, and was not analyzed in our study. However, we cannot exclude that fucoidan may have an additional direct effect on the adhesion of *P. gingivalis* to the ILT, which could also play a role in limiting neutrophil trapping.

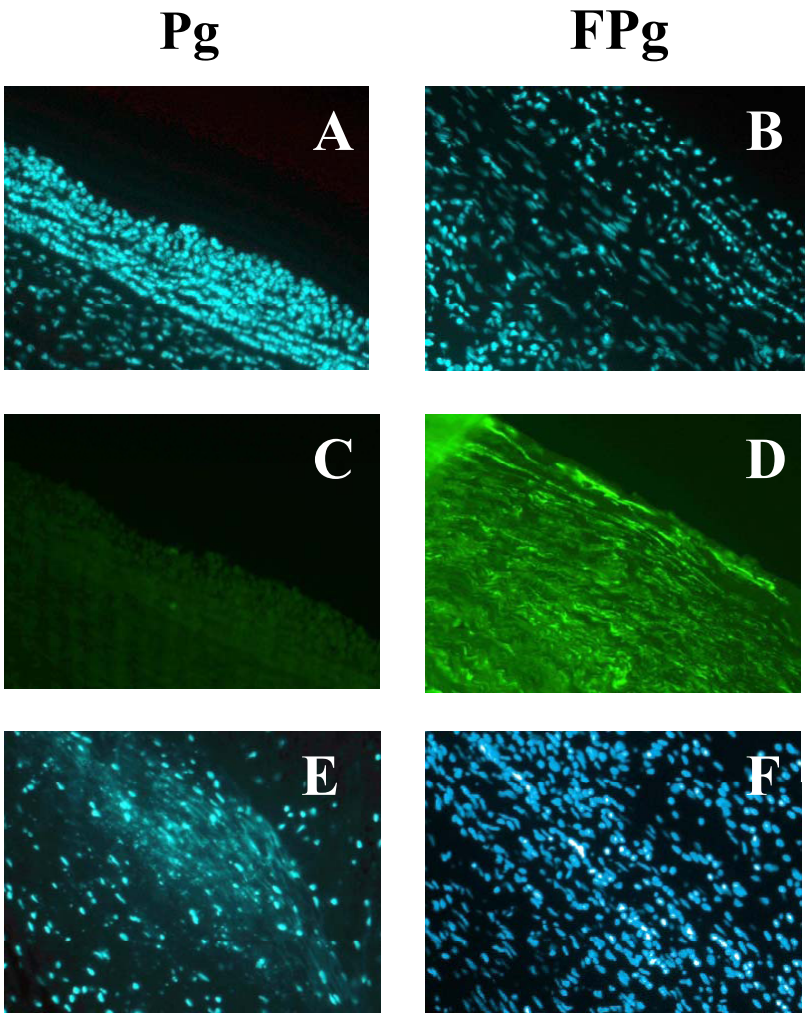

Fig 7. A qualitative indication of neutrophil increased number at the luminal portion of intraluminal thrombus (ILT) in the Porphyromonas gingivalis (*P. gingivalis*; *Pg*) group **(A)** compared to the fucoidan (F)+Pg group **(B)** is confirmed after 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) fluorescence at ×40. Signs of a healing process of the abdominal aortic aneurysm (AAA) treated by fucoidan are confirmed by the high density of actin stained cells into the aortic wall at ×40 of the F+Pg group **(D)** compared to the Pg group **(C)**, suggesting a mesenchymal recolonization. Extranuclear DNA is visualized on DAPI fluorescence ×40, in the ILT of Pg injected AAAs **(E)**, suggesting an enhanced neutrophil activation, whereas only nuclear DNA is seen in the fucoidan-treated aortic wall **(F)**.

Fucoidan has been extensively studied for its diverse biological activities. However, there is no detailed report investigating its toxicity. In a study by Li et al,[41] the acute and chronic (6 months) toxicity of varying levels of fucoidan was investigated in Wistar rats after oral administration.[41] The results showed that no significant toxic changes were observed when 300 mg/kg body weight per day of fucoidan was administered to rats. However, when the dose was increased to 900 and 2500 mg/kg body weight per day, the clotting time was significantly prolonged. Besides this, no other signs of toxicity were observed. Based on these results, it can be concluded that there is no adverse effect level of fucoidan at 300 mg/kg body weight per day.

P. gingivalis were injected at the dose of 10^7 CFU/500 μL/rat, without providing any septic sign, and representing 20 times more than the dose injected of *Staphylococcus aureus* to induce an aortic endocarditis in rats.[42] Pathogens of the subgingival plaque can easily reach the bloodstream several times a day via chewing and brushing teeth, especially in patients with periodontal disease. Chronic bacteremia could, therefore, provide a subclinical infection of various cardiovascular tissues, such as thrombus of the AAA. *P. gingivalis* is a strict anaerobic bacterium that cannot proliferate in the mural thrombus. It is therefore a weak pathogen by itself, but induces neutrophil activation detrimental to the healing of the aneurysm.

Limitations and conclusions. Our study provides experimental data based on a recently developed model of AAAs with a neutrophil-rich ILT relevant to human pathophysiology. However, additional epidemiologic studies

linking AAAs and periodontal diseases would be necessary to support our findings. The model used is a decellularized xenograft transposition characterized by the formation of a thrombus after 1 week. The healing process induced by mesenchymal cell colonization of the aneurysmal wall, that usually takes place thereafter in the absence of additional injury, has been considered to be the main limitation of experimental AAAs in small animals, as this process is not observed in human evolutive AAAs. Now that the implication of weak pathogens, such as *P. gingivalis*, has been described to explain the recurrent enrichment in neutrophils of the ILT, this limitation, represented by the fibrotic cicatrization of the aneurysmal wall, could be considered as a goal to reach in treating human AAAs. Our results suggest that fucoidan induces this reversal effect on experimental aneurysmal degeneration by decreasing Pg-dependent neutrophil activity in the ILT.

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AUTHOR CONTRIBUTIONS

Conception and design: JA, JM, SD, OM
Analysis and interpretation: JA, SD, OM
Data collection: JA, MR, SD, CJ, LL
Writing the article: JA, JM
Critical revision of the article: JM, SD, OM
Final approval of the article: JA, JM
Statistical analysis: SD, JA, CJ
Obtained funding: JM
Overall responsibility: JA, JM, OM

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